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May 14, 2007

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Respectfully submitted,

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FOREIGN PATENT DOCUMENTS

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		Chung, et al; Conformationally Constrained Amino Acids. Synthesis and Optical Resolution of 3-Substituted Proline Derivatives"; *J. Org. Chem.* 55,pp. 270-275 (1990).
		Marki, et al; "The Anticoagulant and Antithrombotic Properties of Hirudins"; *Thrombosis and Haemostasis*; F.K. Schattauer Veriagsgesellschaft mbH (Stuttgart) 64(3) pp. 344-348 (1990).

*Examiner		Date Considered	

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Initial a this form with next communication to application.

J. Org. Chem. 1990, *55*, 270–275

Conformationally Constrained Amino Acids. Synthesis and Optical Resolution of 3-Substituted Proline Derivatives

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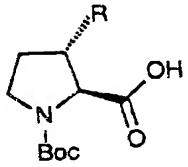
The literature route to racemic cis- and trans-3-substituted prolines starting from acetamidomalonate and α,β-unsaturated aldehydes has been refined and applied to the synthesis of 3-phenyl- and 3-*n*-propylproline. A key improvement in the sequence was the acid-catalyzed silane reduction of the initial hydroxylactam Michael adduct **2**, which allowed subsequent transformations to proceed cleanly and in high yield. Both the *N*- and 3-substitutents were found to have an effect on selectivity in the saponification of trans-3-substituted proline esters in the presence of the corresponding cis esters. The trans isomers in each series were resolved via the diastereomeric (*S*)-α-methylbenzylamides, and the absolute configurations of the resulting pure optical isomers were assigned.

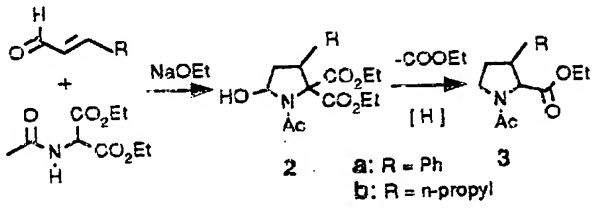
Proline is a well-known means of inducing conformational constraints into peptides.[1] Because of motional restrictions inherent to the pyrrolidine ring, the presence of a proline residue greatly reduces the available conformational space of a peptide and gives rise to conformers separated by relatively high interconversion barriers.[2] Thus, information may be obtained about the bioactive conformation of a peptide, and the biological potency may be increased by incorporation of a proline residue. However, when substitution of an amino acid residue with proline leads to a reduction in biological activity, the question arises whether the loss is due to conformational or steric considerations or to loss of a favorable interaction associated with the side chain of the original amino acid residue. In this vein, we have examined the incorporation of 3-substituted proline derivatives into peptides, where the 3-substituent corresponds to the substituent on the β-carbon of standard amino acids. For example, 3-substituted prolines **1a–c** are conformationally constrained analogues of phenylalanine, norleucine, and aspartate, respectively.



1a, R = Ph
1b, R = $CH_2CH_2CH_3$
1c, R = CO_2R'

In this paper, we describe our work on the synthesis and enantiomeric resolution of derivatives of 3-phenyl- and 3-*n*-propylproline. A stereospecific synthesis of *N*-Boc-*trans*-3-*n*-propyl-L-proline, **1b**, from 4-hydroxy-L-proline was recently developed in our laboratory,[3] but the route was not applicable to the synthesis of **1a** and was not amenable to large-scale work. We were attracted to the schemes outlined by Cox et al.,[4] Mauger et al.,[5] and Sarges et al.[6] for the synthesis of racemic 3-methyl- and 3-phenylproline, which entail condensation of an (acylamino)malonate to the appropriate α,β-unsaturated aldehyde, followed by a sequence of reactions that effect overall 5-deoxygenation and 2-dealkoxycarbonylation (Scheme I). Here we report observations made during our work on the synthesis of 3-phenylproline according to the literature method[6] and describe some refinements in the published procedure. In addition, further modifications were required during the application of these procedures to the synthesis of 3-*n*-propylproline. Finally, we report the optical resolution of the trans isomer of each analogue via the diastereomeric α-methylbenzylamides and the assignment of absolute stereochemistry to the pure optical isomers.

Scheme I



Results and Discussion

The literature reports suggest several procedural variations for effecting conversion of the initial Michael adduct **2** to a mixture of cis- and trans-3-substituted prolines **3** (Scheme I). The major options essentially reduce to whether the reduction in oxidation state at C-5 precedes or follows the dealkoxycarbonylation at C-2. In the reported synthesis of 3-phenylproline,[6] C-5 reduction followed by a standard malonate-type dealkoxycarbonylation sequence gave the cis isomer as the major product, whereas significant quantities of the trans isomer were obtained only when dealkoxycarbonylation preceded C-5 reduction or when the cis ester was saponified under forcing conditions.

During our efforts at replicating these procedures, it became evident that some refinements in the protocol should afford a more attractive method for preparing either or both isomers of the target structure. In particular, deoxygenation at C-5 early in the sequence is clearly preferable in terms of yield and ease of handling, since

(1) (a) Momany, F. A; Chuman, H. *Methods Enzymol.* 1986, *124*, 3. (b) Marshall, G. R. In *Chemical Recognition in Biological Systems*; Creighton, A. M., Turner, S., Eds.; The Chemical Society: London, 1982; p 279. (c) Arison, B. H.; Hirschmann, R.; Veber, D. F. *Bioorg. Chem.* 1978, *7*, 447.

(2) (a) Hollósi, M.; Radics, L.; Wieland, T. *Int. J. Peptide Protein Res.* 1977, *10*, 286. (b) Delaney, N. G.; Madison, V. *Int. J. Peptide Protein Res.* 1982, *19*, 543, and references therein.

(3) Holladay, M. W.; May, C. S.; Arnold, W. A., unpublished results. **1b**: mp 88–90 °C; $[\alpha]_D^{25} = -40.6°$ (*c* 1, $CHCl_3$). Optical purity of this product was assessed as >98% based on HPLC analysis of derived α-methylbenzylamides.

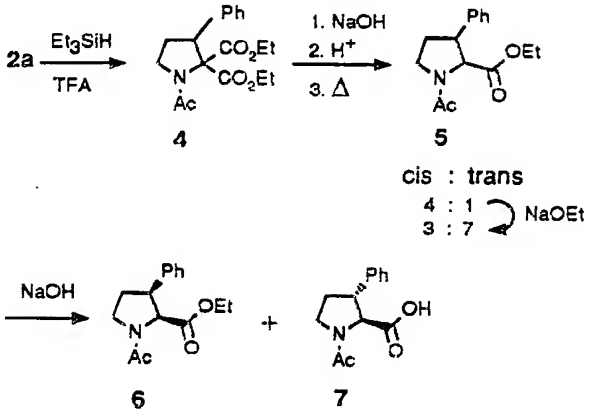
(4) Cox, D. A.; Johnson, A. W.; Mauger, A. B. *J. Chem. Soc.* 1964, 5024.

(5) Mauger, A. B.; Irreverre, F.; Witkop, B. *J. Am. Chem. Soc.* 1966, *88*, 2019.

(6) Sarges, R.; Tretter, J. R. *J. Org. Chem.* 1974, *39*, 1710.

0022-3263/90/1955-0270$02.50/0

Scheme II

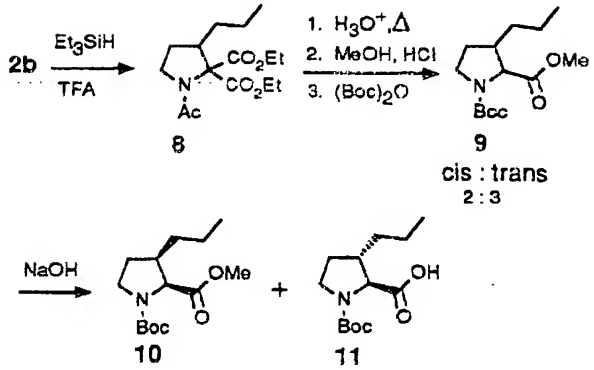


there is significantly greater tendency for the occurrence of side reactions, including retro-Michael reversion of **2** to its educts, while C-5 is still at the aldehyde oxidation state. Moreover, C-5 deoxygenation is readily effected in a single step in high yield using an acid-catalyzed silane reduction procedure.[7] Although the approach involving early C-5 reduction does ultimately lead to a predominance of cis isomer as reported, predominant trans isomer may be readily obtained, if desired, by epimerization of the cis ester under anhydrous conditions.[8]

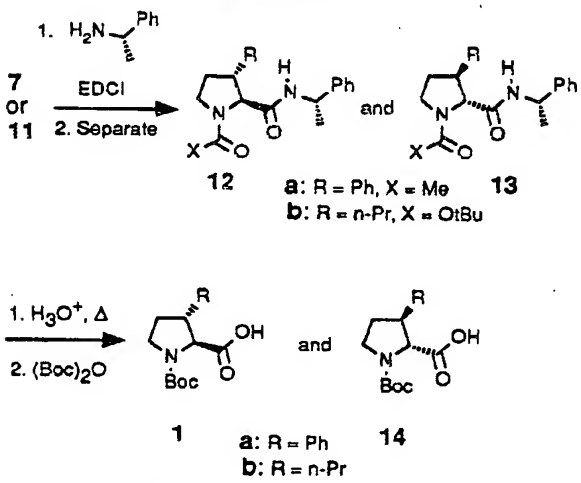
In accord with the above discussion, the sequence described by Scheme II was found to proceed in high yields and in a straightforward fashion. Treatment of **2a** with Et_3SiH/CF_3CO_2H in chloroform[7] provided the pyrrolidine diester **4** (94%), which after monosaponification and decarboxylation was converted to a 1:4 mixture of trans:cis esters **5** in 89% yield. Selective saponification cleanly removed the minor trans isomer as the acid, as reported for the corresponding 4,5-dehydro series,[6] whereas epimerization with NaOEt/EtOH provided a 7:3 equilibrium mixture of trans:cis esters, which were similarly separated by selective saponification to racemic **6** and **7**.

Unexpectedly, an additional modification was required in the 3-*n*-propyl series. In contrast to the 3-phenyl series, mild saponification of a mixture of *cis*- and *trans*-*N*-acetyl-3-*n*-propylproline ethyl esters, **3b**, resulted in partial saponification of the cis ester to afford a 30:70 ratio of cis:trans acids. In contrast, we had found in earlier work[3] on the synthesis of 3-*n*-propyl-L-proline by a different route that the selective saponification of the corresponding mixture of *N*-Boc methyl esters provided a clean separation of the cis and trans isomers. Thus, steric bulk both in the *N*-acyl group and in the 3-substituent apparently contributes to reducing the susceptibility of a cis-3-substituted-proline ester to saponification. To efficiently incorporate the required change of *N*-acyl group into the synthetic route, the modified sequence described in Scheme III was developed. After 5-deoxygenation of **2b** with Et_3SiH/CF_3COOH, the resulting diester **8** was subjected to aqueous acidic conditions at elevated temperature to effect hydrolysis and decarboxylation in a single step, to afford a 2:3 mixture of *cis*- and *trans*-3-*n*-propylproline

Scheme III



Scheme IV



hydrochlorides. Esterification with methanolic HCl followed by protection with a Boc group provided the corresponding mixture of racemic *trans*- and *cis*-3-*n*-propylproline methyl esters **9** in 80% overall yield from **2b**, which were then separated by selective saponification to racemic **10** and **11**.

Among several methods examined for optical resolution of racemic *trans*-3-*n*-propylproline, conversion to the α-methylbenzylamides **12b/13b** followed by recrystallization or chromatographic separation provided the best results (Scheme IV). In the 3-phenyl series, **12a** and **13a** were readily separable by chromatography, but thus far all attempts to crystallize either diastereomer have been unsuccessful. Acid hydrolysis of the pure diastereomeric amides provided the optically pure free amino acids, which after N-protection to **1** and **14** were ready for incorporation into peptides using standard methods.

The absolute stereochemistry of the 3-*n*-propyl series was assigned by correlation to the product synthesized from 4-hydroxy-L-proline[3] and confirmed by single-crystal X-ray analysis of **12b** (Figure 1).

The absolute configuration of the 3-phenyl series was determined by N-deprotection followed by decarboxylation[9] of **1a** and **14a** to the corresponding enantiomers of 3-phenylpyrrolidine, for which absolute stereochemistry has been assigned.[10] Both isomers of 3-phenylpyrrolidine

(7) Auerbach, J.; Zamore, M.; Weinreb, S. M. *J. Org. Chem.* **1976**, *41*, 725.

(8) When we repeated the procedure reported in ref 6 for conversion of *N*-acetyl-2,2-bis(ethoxycarbonyl)-3-phenylpyrrolidine to *trans*-*N*-acetyl-3-phenylproline (NaOH, dioxane/H_2O, 80 °C), the crude product contained a 2:1 mixture of trans:cis acids; one recrystallization from EtOAc/hexane afforded a 6:1 trans:cis mixture in the precipitate fraction, whereas the mother liquors contained a 1:4 trans:cis mixture.

(9) Hashimoto, M.; Eda, Y.; Osanai, Y.; Iwai, T.; Aoki, S. *Chem. Lett.* **1986**, *893*.

(10) (a) Bettoni, G.; Cellucci, C.; Tortorella, V. *J. Heterocycl. Chem.* **1976**, *13*, 1053. (b) Tseng, C. C.; Terashima, S.; Yamada, S.-I. *Chem. Pharm. Bull.* **1977**, *25*, 166.

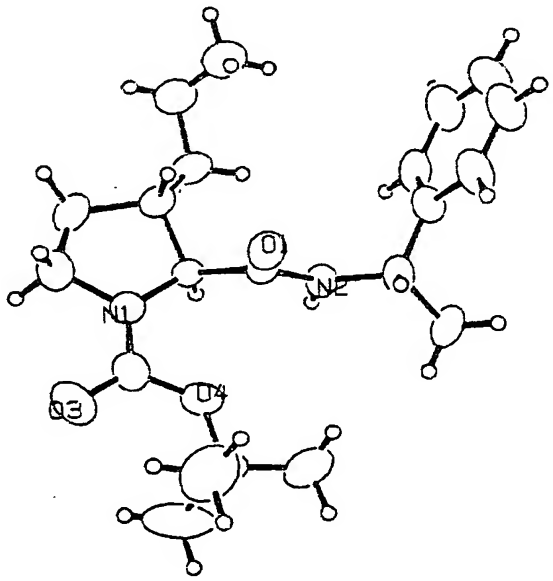


Figure 1. ORTEP drawing of 12b.

were converted to Mosher[13] amide derivatives, which were examined by ^{19}F NMR and determined to have enantiomeric excesses of 86% and 96% for the *R* and *S* isomers, respectively. The low values observed for the optical rotations of the 3-phenylpyrrolidines are not consistent with this analysis and thus presumably were influenced by the presence of optically active impurities. Accordingly, it was desirable to substantiate the stereochemical assignment by appropriate comparisons with an authentic sample of optically active 3-phenylpyrrolidine. The preparation of (*S*)-3-phenylpyrrolidine from (*S*)-2-phenylsuccinic acid was carried out by a modification of the literature method.[10a] The Mosher amide from this product corresponded by ^{19}F NMR to the product obtained from **14a**, which confirms that **14a** has the D-trans stereochemistry.

In summary, literature methods for the preparation of 3-substituted proline derivatives have been refined to provide convenient procedures for the preparation of these analogues.[11] The modifications include the use of Et_3SiH/TFA for the reduction of **2** and the use of a Boc group on **9** for enhanced selectivity during selective saponification of the trans ester in the presence of the cis isomer. The racemic trans isomers of 3-phenyl- and 3-*n*-propylproline were resolved, and the absolute configurations of optically pure derivatives were assigned. The overall sequence was easily carried out on a 200-g scale, which makes these analogues readily available for peptide studies. Application of this method to other 3-substituted prolines is currently underway.[12]

Experimental Section

Proton magnetic resonance spectra were obtained on a Nicolet QE-300 (300 MHz) or a General Electric GN-300 (300 MHz) instrument. Chemical shifts are reported as δ values (ppm) relative to Me_4Si as an internal standard unless otherwise indicated. Spin–spin decoupling and NOE difference spectra were obtained on a General Electric GN-500 (500 MHz) instrument. Mass spectra were obtained with Hewlett-Packard HP5965 (CI) and Kratos MS50 (FAB, HRMS) spectometers. Infrared spectra were recorded on a Perkin-Elmer 283 spectrophotomer. Elemental analyses and the above determinations were performed by the Analytical Research Department, Abbott Laboratories.

Thin-layer chromatography (TLC) was carried out using E. Merck precoated silica gel F-254 plates (thickness 0.25 mm). Preparative thin-layer chromatography (PTLC) was carried out using Analtech 20 × 20 cm precoated silica gel GF plates (thickness 1.00 mm). Flash column chromatography was performed on Merck Silica Gel 60, 200–400 mesh.

Melting points are uncorrected and were determined on either a Thomas-Hoover or a Büchi 510 melting point apparatus. Optical rotation data were obtained on Perkin-Elmer Model 241 polarimeter. Anhydrous solvents were purchased from Aldrich (Milwaukee, WI), and reactions requiring anhydrous solvents were performed under a nitrogen atmosphere.

Abbreviations used are as follows: HOBt, 1-hydroxybenzotriazole; EDCI, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride.

Diethyl 1-Acetyl-3-phenylpyrrolidine-2,2-dicarboxylate (4). To a solution of **2a**[4] (4.35 g, 12.4 mmol) and triethylsilane (3.0 mL, 18.6 mmol) in 25 mL of $CHCl_2$ was added trifluoroacetic acid (9.5 mL, 124 mmol) dropwise with stirring over 10 min. After stirring for 2.5 h at room temperature, the solution was concentrated in vacuo, and then an EtOAc solution of the residue was washed with aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated to 4.3 g of oily residue, which was sufficiently pure for direct use. A 444-mg sample was distilled (Kugelrohr: 206–214 °C bath temperature, 0.7 mmHg) to afford 400 mg (94%) of pale yellow oil: ^1H NMR ($CDCl_3$) δ 0.85 (t, J = 7.5 Hz, 3 H), 1.30 (t, J = 7.5 Hz, 3 H), 2.15 (s, 3 H), 2.25 (quintet of doublets, J_1 = 1.8 Hz, J_2 = 6 Hz, 1 H), 2.60 (m, 1 H), 3.75 (m, 2 H), 3.90 (m, 3 H), 4.30 (q, J_1 = 7.5 Hz, 2 H), 7.20 (m, 2 H), 7.30 (m, 3 H); HRMS calcd for $C_{18}H_{23}NO_5$ m/e 333.1576, found m/e 333.1569.

***N*-Acetyl-3-phenylproline Ethyl Ester (5, Mixture of Cis and Trans Isomers).** Crude diester **4** (1.8 g, 5.4 mmol) was suspended in 50 mL of 0.5 N NaOH and stirred at room temperature for 21 h. The resultant solution was extracted once with EtOAc and then acidified to pH 2 with 3 N HCl. The precipitate was extracted into $CHCl_3$, and the aqueous phase twice extracted with $CHCl_3$. The combined $CHCl_3$ fractions were concentrated to 1.58 g of solid residue, which was heated for 1 h at 75 °C in 20 mL of toluene. Evaporation of the solvent afforded 1.2 g of **5** (cis:trans = 4:1): MS (CI), m/e 262 ($(M + H)^+$); partial ^1H NMR ($CDCl_3$) cis isomer (two conformers) δ 0.73 (t, J = 7.5 Hz, 2.2 H), 0.82 (t, J = 7.5 Hz, 0.8 H), 4.56 (d, J = 8.4 Hz, 0.2 H), 4.62 (d, J = 9 Hz, 0.8 H), trans isomer two conformers) δ 1.20 (t, J = 7.5 Hz, 2.1 H), 1.28 (t, J = 7.5 Hz, 0.8 H), 4.39 (d, J = 4.5 Hz, 0.25 H), 4.52 (d, J = 6 Hz, 0.75 H). Anal. Calcd for $C_{15}H_{19}HO_3 \cdot 0.1H_2O$: C, 68.47; H, 7.35; N, 5.32. Found: C, 68.42; H, 7.35; N, 5.29.

***N*-Acetyl-*trans*-3-phenylproline (7).** A total of 950 mg (3.6 mmol) of **5** was heated at reflux for 2 h with 22 mL of 1 M NaOEt in EtOH, which contained 430 μL of ethyl trifluoroacetate as an H_2O scavenger. After cooling, water (15 mL) was added, the solution was stirred for 2.5 h at ambient temperature, and then the acidic and neutral components were separated by standard extractive techniques to afford 664 mg (78%) of **7** and 200 mg (21%) of cis-ester **6**. Recrystallization of 135 mg of crude **7** from MeOH/EtOAc afforded 112 mg of analytically pure material: mp 182–184 °C (lit.[4] mp 180–181 °C); MS (CI), m/e 251 ($(M + NH_4)^+$), 234 ($(M + H)^+$); ^1H NMR (Me_2SO-d_6, two conformers) δ 1.89 (s, 0.7 H), 2.0–2.1 (cmplx, 3.1 H, includes 2.02 (s, 2.3 H)), 2.18 (m, 0.2 H), 2.30 (m, 1 H), 3.38 (m, 1 H, partially obscured by H_2O), 3.60 (m, 1 H), 3.73 (m, 1 H), 4.15 (d, J = 6 Hz, 0.8 H), 4.98 (d, J = 6 Hz, 0.2 H). Anal. Calcd for $C_{13}H_{15}NO_3 \cdot 0.1MeOH$: C, 66.54; H, 6.56; N, 5.92. Found: C, 66.42; H, 6.36; N, 5.85. Partial ^1H NMR spectrum for **6** ($CDCl_3$) δ 0.64 (t, J = 7.5 Hz, 2.25 H), 0.83 (t, J = 7.5 Hz, 0.75 H), 2.0 (s, 0.75 H), 2.12 (s, 2.25 H), 4.56 (d, J = 8 Hz, 0.25 H), 4.73 (d, J = 9 Hz, 0.75 H).

Preparation and Separation of 12a and 13a. A solution of **7** (917 mg, 3.92 mmol), $HOBt \cdot H_2O$ (659 mg, 4.31 mmol), and (*S*)-(−)-α-methylbenzylamine (555 μL, 521 mg, 4.31 mmol) in 10

(11) After a substantial amount of this work had been completed, publication of the preparation of 3-substituted prolines from a glycine–nickel complex and α,β-unsaturated aldehydes became known: Belokon', Y. N.; Bulychev, A. G.; Pavlov, V. A.; Fedorova, E. B.; Tsyryapkin, V. A.; Bakhmutov, V. A.; Belikov, V. M. *J. Chem. Soc., Perkin Trans. 1* 1988, 2075.

(12) In a preliminary study, treatment of the methyl ester of **1a** with RuO_4 gave methyl *N*-[(*tert*-butyloxy)carbonyl]-*trans*-3-carboxypyroglutamate in good yield.

(13) Dale, J. A.; Dull, D. A.; Mosher, H. S. *J. Org. Chem.* 1969, *34*, 2543.

mL of DMF was cooled to 0° C and then EDCI (823 mg, 4.31 mmol) was added. The mixture was allowed to warm to room temperature while stirring for 3 h and then was diluted with EtOAc and 1 M $KHSO_4$. The layers were separated, and the organic phase was washed successively with H_2O, saturated aqueous $NaHCO_3$, and brine, then dried (Na_2SO_4), and concentrated. The residue was chromatographed over 125 g of silica gel (1.5% HOAc/EtOAc) to provide 562 mg (42%) of **12a** (97:3 mixture with **13a** by ^1H NMR) and 655 mg (50%) of **13a** (>98% diastereomeric purity by ^1H NMR). **12a**: R_f = 0.24 (2.5% HOAc/EtOAc); $[\alpha]^{24}_D$ = +13.8° (*c* 0.88, MeOH); MS (CI) *m/e* 337 ((M + H)$^+$), 188; ^1H NMR ($CDCl_3$) δ 1.45 (d, *J* = 7 Hz, 3 H), 2.1 (m, 1 H), 2.19 (s, 3 H), 2.5 (m, 1 H), 3.67 (t, *J* = 7.5 Hz, 2 H), 3.82 (m, 1 H), 4.51 (d, *J* = 4.5 Hz, 1 H), 5.05 (m, 1 H), 7.0 (br d, *J* = 7.5 Hz, 1 H), 7.1–7.35 (cmplx, 10 H). **13a**: R_f = 0.40 (2.5% HOAc/EtOAc); $[\alpha]^{24}_D$ = −147.8° (*c* 0.68, MeOH); MS (CI) *m/e* 337 ((M + H)$^+$), 188; ^1H NMR ($CDCl_3$) δ 1.42 (d, *J* = 6 Hz, 2.5 H), 1.47 (d, *J* = 7.5 Hz, 0.5 H), 2.05 (m, 1 H), 2.08 (s, 0.5 H), 2.15 (s, 2.5 H), 2.5 (m, 1 H), 3.6 (m, 2 H), 3.93 (m, 1 H), 4.25 (d, *J* = 4.5 Hz, 0.2 H), 4.61 (d, *J* = 4.5 H, 0.8 H), 5.05 (m, 0.8 H), 5.20 (m, 0.2 H), 7.15–7.38 (complex, 11 H).

***N*-[(*tert*-Butyloxy)carbonyl]-*trans*-3-phenyl-L-proline (1a).** Amide **12a** (528 mg, 1.57 mmol, 97:3 mixture with **13a**) was dissolved in 4 mL of HOAc and 12 mL of 8 N HCl and heated at reflux for 18 h. The mixture was concentrated to dryness, the residue was taken up in H_2O and extracted once with an equal volume of EtOAc, and the aqueous phase was again concentrated to dryness. The residue was dissolved in 8 mL of 1:1 H_2O/dioxane and treated with NEt_3 (484 μL, 353 mg, 3.50 mmol) and di-*tert*-butyl dicarbonate (753 mg, 3.45 mmol). After stirring for 4 h, additional NEt_3 was added to ca. pH 9, then stirring was continued for an additional 18 h. Extractive isolation of the acidic component afforded 313 mg of a tan foam. Chromatography over silica gel (1:1 EtOAc/hexane, containing 1% HOAc) afforded 260 mg (57%) of **1a**: mp 158–160 °C; $[\alpha]^{24}_D$ = +33.7° (*c* 1.0, $CHCl_3$); MS (CI), *m/e* 309 ((M + NH_4)$^+$), 292 ((M + H)$^+$), 253, 236, 192, 146; ^1H NMR ($CDCl_3$) (two conformers) δ 1.43 (s, 4 H), 1.52 (s, 5 H), 2.03 (m, 1 H), 2.33 (m, 1 H), 3.50 (m, 1 H), 3.6–3.8 (complex, 1.45 H), 3.87 (m, 0.55 H), 4.30 (d, *J* = 6.3 Hz, 0.45 H), 4.45 (d, *J* = 4.8 Hz, 0.55 H), 7.2–7.4 (complex, 5 H). Anal. Calcd for $C_{16}H_{21}NO_4$: C, 65.95; H, 7.27; N, 4.81. Found: C, 65.81; H, 7.27; N, 4.73.

***N*-[(*tert*-Butyloxy)carbonyl]-*trans*-3-phenyl-D-proline (14a)** was prepared similarly from **13a** (453 mg, 1.34 mmol, >98% diastereomeric purity), except in this case, purification of 320 mg of crude product was effected by recrystallization from EtOAc/hexane to afford 182 mg (47%) of **14a**: mp 162–163 °C; $[\alpha]^{24}_D$ = −35.9° (*c* 1.0, $CHCl_3$). Found: C, 66.10; H, 7.31; N, 4.78. NMR and MS data were identical with those for **1a**. A second crop of 56 mg (14%), mp 159–160 °C, was obtained from the mother liquors.

(*S*)-(+)-3-Phenylpyrrolidine. a. The Boc group was removed from acid **14a** (91 mg, 0.31 mmol) by treatment with 1:1 CH_2Cl_2/CF_3CO_2H for 1 h at room temperature. The volatile components were evaporated under reduced pressure, and then the residue was dissolved in 1 mL of H_2O and applied to a column (2-mL bed volume) of Dowex-50 (H^+ form). The column was washed with H_2O, and then the amino acid was eluted with 2 N NH_4OH. Combined ninhydrin-positive fractions were lyophilized to give 63 mg of the free amino acid in zwitterionic form: ^1H NMR (D_2O) δ 2.22 (m, 1 H), 2.50 (m, 1 H), 3.51 (m, 1 H), 3.61 (m, 1 H), 4.12 (d, *J* = 9 Hz, 1 H), 7.45 (m, 5 H). A 59-mg sample of this product was heated with stirring under N_2 in a 160 °C oil bath in the presence of 1 mL of cyclohexanol and 5 μL of 2-cyclohexen-1-one until the solid had dissolved (ca. 15 min.). After the solution had cooled, it was treated with 5 mL each of H_2O and Et_2O, the aqueous phase was made acidic with 3 N HCl, and then the layers were separated. The aqueous phase was washed with three additional portions of Et_2O and then was made basic by addition of solid K_2CO_3. The resulting solution was continuosuly extracted with Et_2O, and then the organic phase was dried over $MgSO_4$ and evaporated to 49 mg of a pale yellow residue, which was distilled by using a microsublimation apparatus. After collection of a forerun fraction at 60 °C (bath temperature) and 4.2 mmHg, a second fraction (8.9 mg) was collected at 105 °C (bath temperature) and 4.2 mmHg; MS (CI), *m/e* 148 ((M + H)$^+$); ^1H NMR ($CDCl_3$) δ 1.95 (m, 1 H), 2.31 (m, 1 H), 2.89 (br m, NH), 2.98 (t, *J* = 10 Hz, 1 H), 3.21 (m, 1 H), 3.35 (m, 2 H), 3.50 (dd, *J* = 7.5, 10 Hz, 1 H), 7.28 (m, 5 H); $[\alpha]^{24}_D$ = +16.0° (*c* 0.04, MeOH); $[\alpha]^{24}_D$ = +13.8° (*c* 0.48, EtOH), lit. for *R* isomer $[\alpha]_D$ = −24° (*c* 4.7%, MeOH),[10a] for the *S* isomer $[\alpha]^{20}_D$ = +22.7° (*c* 2.36, EtOH).[10b] The (*α*-methoxy-*α*-(trifluoromethyl)phenyl)acetyl (MTPA) amide was prepared by condensation with (−)-MPTA chloride in CCl_4/pyridine;[13] MS (CI), *m/e* 364 ((M + H)$^+$); ^{19}F NMR (Me_2SO-d_6, 145 °C) (ppm, downfield relative to the center peak of the trifluoroethanol triplet) δ 5.82 (s), 6.18 (s) in a ratio of 45:1.

b. (*S*)-*N*-Benzyl-3-phenylpyrrolidine was prepared from (*S*)-(+)-2-phenylsuccinic acid by a procedure similar to that described.[10a] Debenzylation was carried out by catalytic hydrogenolysis over 20% PdC in MeOH (4 atm, room temperature). After removal of the catalyst by filtration, the solvent was evaporated to provide the crude product, which was confirmed by comparison of the ^1H NMR spectrum with that from (a). ^{19}F NMR of the product from (−)-MPTA chloride (same conditions as above) δ 5.82 (s).

(*R*)-(−)-3-Phenylpyrrolidine was obtained from **1a** in a manner analogous to that described for the preparation of the *S* isomer from **14a**; ^1H NMR and MS data were identical with those for the *S* isomer; $[\alpha]^{23}_D$ = −12.4° (*c* 0.9, MeOH); $[\alpha]^{24}_D$ = −14.6° (*c* 0.6, EtOH); ^{19}F NMR of the MTPA amide from (−)-MTPA chloride (same conditions as above) δ 5.82 (s), and 6.12 (s) in a ratio of 1:13.

Diethyl 1-Acetyl-5-hydroxy-3-*n*-propylpyrrolidine-2,2-dicarboxylate (2b). Sodium (3.48 g, 0.15 mol) was dissolved in a stirred solution of diethyl acetamidomalonate (201.6 g, 0.93 mol) in anhydrous ethanol (1200 mL) at room temperature under nitrogen. The reaction mixture was cooled to 0 °C, and *trans*-2-hexenal (100.0 g, 1.02 mol) was then added dropwise. The resulting mixture was allowed to warm to room temperature. After stirring for 3 h at 23 °C, the reaction was quenched with 24 mL of acetic acid. The solution was concentrated in vacuo, and the resulting residue was taken up in EtOAc and washed successively with saturated aqueous $NaHCO_3$ (2×) and brine, then dried over $MgSO_4$, and concentrated. The residue was crystallized from EtOAc/hexane to give 271.4 g (93%) of **2b** as fine needles: mp 105–106 °C; TLC R_f = 0.43 (9:1 $CHCl_3$/MeOH); IR ($CDCl_3$) 3460, 1750, 1665 cm^{-1}; MS(CI), *m/e* 316 ((M + H)$^+$), 298 ((MH − H_2O)$^+$); ^1H NMR (CD_3OD) δ 5.64 (d, *J* = 5.2 Hz, 1 H), 4.26–4.10 (m, 4 H), 2.83 (m, 1 H), 2.21 (s, 3 H), 2.08 (dd, *J* = 12.8, 6.4 Hz, 1 H), 1.94 (dd, *J* = 12.8, 5.2 Hz, 1 H), 1.90–1.78 (m, 1 H), 1.47–1.11 (m, 9 H), 0.94 (t, *J* = 7.2 Hz, 3 H). Anal. Calcd for $C_{15}H_{25}NO_6$: C, 57.13; H, 7.99; N, 4.44. Found: C, 57.28; H, 8.08; N, 4.42.

Diethyl 1-Acetyl-3-*n*-propylpyrrolidine-2,2-dicarboxylate (8). To a solution of **2b** (271.0 g, 0.86 mol) and triethylsilane (206 mL, 1.29 mol) in CH_2Cl_2 (3 L) was added trifluoroacetic acid (663 mL, 8.6 mol) dropwise with stirring while controlling the internal temperature at 25–30 °C by means of an ice bath. After stirring for 3 h at 23 °C, the solution was concentrated in vacuo, and the residue was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ solution until all the TFA was neutralized. The organic phase was dried (Na_2SO_4) and concentrated to give 350 g of oil. The oily product containing silicon impurities was used directly in the subsequent step. An analytically pure sample was obtained as an oil after flash chromatography (EtOAc/hexane, 1:2, then 1:1): TLC, R_f = 0.55 (9:1 $CHCl_3$/MeOH); IR ($CDCl_3$) 1745, 1655 cm^{-1}; MS(CI), *m/e* 300 ((M + H)$^+$); ^1H NMR ($CDCl_3$) δ 4.28–4.17 (m, 4 H), 3.76 ("dt", *J* = 9.6, 1.1 Hz, 1 H), 3.57 (ddd, *J* = 10.7, 9.6, 6.2 Hz, 1 H), 2.51–2.4 (m, 1 H), 2.16–2.01 (m, 4 H), 1.85–1.70 (m, 2 H), 1.42–1.15 (m, 9 H), 0.93–0.89 (t, *J* = 7.0 Hz, 3 H). Anal. Calcd for $C_{15}H_{25}NO_5$: C, 60.18; H, 8.42; N, 4.68. Found: C, 59.90; H, 8.34; N, 4.65.

3-*n*-Propylproline Hydrochloride (Cis:Trans Mixture). The crude ester 8 (350 g) was suspended in 6 N HCl (2 L) and acetic acid (500 mL) and heated at reflux for 17 h. The reaction mixture was extracted with EtOAc (2×), and the aqueous phase was concentrated on a rotary evaporator. The residue was then triturated with ether to crystallize the product. The solid was collected by filtration, washed with ether, and dried in a vacuum oven to give 152.3 g of the hydrochloride salt. An analytically pure sample was obtained by recrystallization from acetone/ether; mp 131–133 °C; TLC R_f = 0.26 (10:4:1 $CHCl_3$/MeOH/NH_4OH);

IR (KBr) 3420, 1735 cm^{-1}; MS (CI), *m/e* 158 (free base M + H^+); ^1H NMR (D_2O) major isomer δ 3.93 (d, J = 7.4 Hz, 1 H), 3.58–3.29 (m, 2 H), 2.50–2.43 (m, 1 H), 2.32–2.16 (m, 1 H), 1.90–1.68 (m, 2 H), 1.50–1.18 (m, 3 H), 0.91 (t, J = 7.2 Hz, 3 H), minor isomer δ 4.32 (d, J = 8.1 Hz, 1 H), 3.58–3.29 (m, 2 H), 2.66–2.60 (m, 1 H), 2.32–2.16 (m, 1 H), 1.90–1.68 (m, 2 H), 1.50–1.18 (m, 3 H), 0.89 (t, J = 6.8 Hz, 3 H). Anal. Calcd for $C_9H_{16}NO_2Cl$: C, 49.61; H, 8.33; N, 7.23. Found: C, 49.35; H, 8.17; N, 7.18.

***N*-[(*tert*-Butyloxy)carbonyl]-3-*n*-propylproline Methyl Ester (9).** The hydrochloride salt (152.3 g) from the above reaction was dissolved in MeOH (1.5 L), and the solution was charged with HCl gas until it was saturated. After stirring overnight, the reaction mixture was concentrated to give an oil. This was taken up in 1 N HCl and extracted with EtOAc (2×). The aqueous layer was carefully made basic with K_2CO_3 and extracted with $CHCl_3$ exhaustively. The combined organic phases were dried ($MgSO_4$), filtered, and concentrated to give 122 g of oil: TLC R_f = 0.70 (90:10:0.1 $CHCl_3$/MeOH/NH_4OH); MS (CI), *m/e* 272 ((M + H)$^+$); partial ^1H NMR ($CDCl_3$) major isomer δ 3.74 (s, 3 H), 3.36 (d, J = 6.3 Hz, 1 H), minor isomer δ 3.83 (d, J = 8.1 Hz, 1 H), 3.72 (s, 3 H).

The oil (122 g) was taken up in MeOH (1 L), and then $NaHCO_3$ (180 g) and di-*tert*-butyl dicarbonate (171.4 g, 0.79 mol) were added slowly. After stirring overnight at 23 °C, the mixture was filtered, and the filtrate concentrated. The residue was triturated with EtOAc, filtered again, and concentrated to give 186.1 g of oil as mixture of cis and trans isomers: TLC R_f = 0.31 (1:6 EtOAc/hexane); IR ($CDCl_3$) 1700, 1745 cm^{-1}; MS (CI), *m/e* 272 ((M + H)$^+$); partial ^1H NMR ($CDCl_3$) cis isomer (two conformers) δ 4.32 (d, J = 8.1 H, 0.4 H), 4.23 (d, J = 8.5 Hz, 0.6 H), 3.72 (s, 1.2 H), 3.71 (s, 1.8 H), 0.91 (t, J = 7.0 Hz, 3 H), trans isomer (two conformers) δ 3.94 (d, J = 5.5 Hz, 0.4 H), 3.81 (d, J = 6.2 Hz, 0.6 H), 3.74 (s, 1.2 H), 3.73 (s, 1.8 H), 3.68–3.56 (m, 1 H), 3.50–3.42 (m, 1 H), 2.25–2.13 (m, 1 H), 2.03–1.92 (m, 1 H), 1.78–1.06 (m, 14 H), 0.92 (t, J = 7.0 Hz, 3 H). Anal. Calcd for $C_{14}H_{25}NO_4$: C, 61.97; H, 9.29; N, 5.16. Found: C, 61.93; H, 9.30; N, 5.11.

***N*-[(*tert*-Butyloxy)carbonyl]-*trans*-3-*n*-propylproline (11) and *N*-[(*tert*-Butyloxy)carbonyl]-*cis*-3-*n*-propylproline Methyl Ester (10).** To a solution of 9 (186.0 g, 0.685 mol) in MeOH (685 mL) was added 1 N NaOH (685 mL) at 23 °C. After stirring for 20 h, the solution was concentrated to remove MeOH and then extracted with EtOAc (3×). The extracts were dried ($MgSO_4$), filtered, and concentrated to give 71.4 g (38%) of cis ester 10: TLC R_f = 0.54 (1:4 EtOAc/hexane); IR ($CDCl_3$) 1745, 1700 cm^{-1}; HRMS (CI), *m/e* 272.1869 ((M + H)$^+$, for $C_{14}H_{26}NO_4$, calcd 272.1862); ^1H NMR ($CDCl_3$) cis isomer (two conformers) δ 4.32 (d, J = 8.1 Hz, 0.4 H), 4.23 (d, J = 8.5 Hz, 0.6 H), 3.72 (s, 1.2 H), 3.71 (s, 1.8 H), 3.68–3.56 (m, 1 H), 3.34–3.25 (m, 1 H), 2.41–2.25 (m, 1 H), 2.12–1.90 (m, 1 H), 1.78–1.64 (m, 1 H), 1.45–1.23 (m, 12 H), 1.20–1.07 (m, 1 H), 0.91 (t, J = 7.0 Hz, 3 H). The aqueous phase was acidified with solid citric acid and extracted 2× with EtOAc. The combined extract was washed with H_2O and brine, dried ($MgSO_4$), filtered, and concentrated to give 94.2 g (53%) of trans-acid 11: TLC R_f = 0.45 (9:1 $CHCl_3$/MeOH); IR ($CDCl_3$) 3050, 1720, 1690 cm^{-1}; MS (CI), *m/e* 258 ((M + H)$^+$); ^1H NMR ($CDCl_3$) (two conformers) δ 3.98 (br d, J = 4.4 Hz, 0.6 H), 3.84 (br d, J = 6.2 Hz, 0.4 H), 3.64–3.34 (m, 2 H), 2.50 (m, 0.6 H), 2.30 (m, 0.4 H), 2.12–1.97 (m, 1 H), 1.69–1.28 (m, 14 H), 0.93 (m, 3 H). Anal. Calcd for $C_{13}H_{23}NO_4$: C, 60.68; H, 9.01; N, 5.44. Found: C, 60.66; H, 8.91; N, 5.44.

Preparation and Separation of 12b and 13b. To a solution of 11 (94.2 g, 0.37 mol) and HOBt·H_2O (54.4 g, 0.40 mol) in CH_2Cl_2 (1 L) was added EDCI (76.9 g, 0.40 mol) under nitrogen at 0 °C. After stirring overnight at 23 °C, the reaction mixture was diluted with EtOAc, washed with 10% aqueous citric acid, saturated aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$, filtered, and concentrated. The residue was dissolved in a minimum amount of ether, and **12b** preferentially crystallized out upon standing (34.0 g, 9:1 **12b**/**13b**). The mother liquor was concentrated, and the resulting residue was chromatographed on silica gel using 35% EtOAc/hexane as eluant to provide fractions containing 46.5 g of **13b** (35%, contains <5% **12b**), 6.9 g of a ca. 1:1 mixture, and 16.3 g of 9:1 **12b**/**13b**. The mixed fraction (6.9 g) and the fractions enriched in **12b** (total 50.3 g) were combined and recrystallized three times from ether/hexane to give a total of 40.2 g (30%) of **12b** (>99% pure). Alternatively, the mixture could be separated by preparative HPLC with about 80% recovery. **12b**: mp 112–114 °C (Et_2O/hexane); TLC, R_f = 0.39 (EtOAc:hexane = 1:2); $[\alpha]^{23}_D$ = −56.3° (*c* 0.75, MeOH); MS (CI), *m/e* 361 ((M + H)$^+$, base), 305, 261, 112; ^1H NMR (Me_2SO-d_6, 138 °C) δ 7.65–7.15 (m, 5 H), 4.99 ("q", J = 7.1 Hz, 1 H), 3.77 (d, J = 5.1 Hz, 1 H), 3.55–3.30 (m, 2 H), 2.14 (m, 1 H), 1.97 (m, 1 H), 1.55–1.25 (m, 5 H), 1.43 (d, J = 7.1 Hz, 3 H), 1.40 (s, 9 H), 0.88 (br t, J = 7.0 Hz, 3 H). Anal. Calcd for $C_{21}H_{32}N_2O_3$: C, 69.97; H, 8.95; N, 7.77. Found: C, 69.99; H, 9.05; N, 7.73.

13b: mp 104–105 °C (Et_2O/hexane); TLC R_f = 0.45 (EtOAc:hexane = 1:2); $[\alpha]^{25}_D$ = −65.8° (*c* 0.76, MeOH); MS (CI) *m/e* 361 ((M + H)$^+$, base), 305, 261, 112; ^1H NMR (Me_2SO-d_6, 138 °C) δ 7.60–7.17 (m, 5 H), 4.98 ("q", J = 7.1 Hz, 1 H), 3.79 (d, J = 4.9 Hz, 1 H), 3.51–3.33 (m, 2 H), 2.18 (m, 1 H), 2.00 (m, 1 H), 1.58–1.45 (m, 2 H), 1.43 (d, J = 7.1 Hz, 3 H), 1.42–1.28 (m, 3 H), 1.36 (s, 9 H), 0.91 (br t, J = 7.0 Hz, 3 H). Anal. Calcd for $C_{21}H_{32}N_2O_3$: C, 69.97; H, 8.95; N, 7.77. Found: C, 70.27; H, 9.25; N, 7.73.

X-ray Crystallographic Absolute Stereostructure Determination of 12b. Colorless single crystals suitable for the collection of X-ray diffraction data were obtained by recrystallization from a solution of 8:1:1 acetonitrile:acetone:water by vapor diffusion against 9:1 water:acetone. A crystal (dimensions 0.25 × 0.02 × 0.90 mm) was selected for data collection and mounted on a Rigaku AFC5 automated four-circle diffractometer. The crystal was found to be orthorhombic, and unit cell parameters and the orientation matrix were obtained. Data collection was carried out using the ω profile mode with real-time Lehmann–Larsen profile analysis: formula $C_{21}H_{32}N_2O_3$; formula weight, 360.50; space group $P2_12_12_1$; a = 12.685 (4) Å; b = 17.094 (4) Å; c = 9.829 (2) Å; V = 2125.2 (8) Å^3; Z = 4; diffractometer, Rigaku AFC5; radiation, Cu Kα; filter, Ni; scan type, ω profile with real-time Lehmann–Larsen profile analysis; scan speed, 1°/min; scan range, 1.5 °; 2θ scan limits, 5–90°; 3 per 150 reflections; indices (−3,1,0), (−3,−1,0), 3,−1,1); crystal stability, standard reflections increased 1.2% during data collection; total reflections scanned, 1033; unique reflections [$I > 3\sigma(I)$] = 788; R, $(\sum||K|F_o| - |F_c||)/(\sum K|F_o|)$ = 0.047; R_w ($\omega = 1/\sigma^2$) 0.058. The data were corrected for absorption, Lorentz, and polarization factors. The structure was solved by SHELXS, a direct-methods program. The structure was refined through the least-squares procedure with the complete matrix of normal equations. Non-hydrogen atoms were refined anisotropically. The hydrogen atom position on N2 was found by fourier difference all other hydrogen atom positions were calculated. The largest and the smallest peaks in the final difference map were +0.174 and −0.133 e/Å^3. (List of positional and anisotropic thermal parameters of non-hydrogen atoms, positional and thermal parameters of hydrogen atoms, bond distances, and bond angles are available as supplementary material (see paragraph at end of paper)).

***N*-[(*tert*-Butyloxy)carbonyl]-*trans*-3-*n*-propyl-L-proline (1b).** A solution of **12b** (40.2 g, 0.11 mol, >95% diastereomerically pure) in 8 N HCl (870 mL) and glacial acetic acid (220 mL) was heated at reflux overnight. The solution was concentrated on a rotary evaporator, and the residue taken into H_2O and extracted with ether. The aqueous phase was concentrated and azeotroped 3× with toluene to give 43.0 g of a mixture of *trans*-3-*n*-propyl-L-proline hydrochloride and α-methylbenzylamine hydrochloride. The salts were taken up in dioxane/H_2O (1:1, 400 mL) and then treated carefully with *N,N*-diisopropylethylamine (35.5 g, 0.275 mol) and di-*tert*-butyl dicarbonate (60.0 g, 0.275 mol) sequentially at 0 °C. After stirring overnight at 23 °C, the mixture was diluted with EtOAc, and the two layers were separated. The organic layer was extracted with 0.5 N NaOH (2×). The combined aqueous layers were extracted with EtOAc once, and the separated organic layer was back-extracted with 0.5 N NaOH. The combined aqueous fractions were cooled to 0–5 °C and acidified to pH 1.0 with cold 4 N HCL and extracted immediately with EtOAc (2×). The combined EtOAc extracts were washed with brine, dried ($MgSO_4$), and concentrated. The residue was dried under reduced pressure over P_2O_5 to give 26.1 g (92%) of **1b** as a white solid: mp 88–89 °C (hexane); TLC, R_f = 0.15 (9:1 $CHCl_3$/MeOH); $[\alpha]^{24}_D$ = −38.3° (*c* 1.0, $CHCl_3$); $[\alpha]^{25}_D$ = −42.5° (*c* 0.095, $CHCl_3$); MS (CI) *m/e* 258 ((M + H)$^+$), 219 (base), 202, 158; ^1H NMR (360 mHz, Me_2SO-d_6, 100 °C) δ 3.67 (d, J = 4.0 Hz, 1 H), 3.40 (m, 1 H), 3.30 (m, 1 H), 2.20 (m, 1 H), 1.98 (m, 1

H), 1.55–1.20 (m, 15 H), 0.90 (t, J = 7.0 Hz, 3 H). Anal. Calcd for $C_{18}H_{23}NO_4$: C, 60.68; H, 9.01; N, 5.44. Found: C, 60.85; H, 8.97; N, 5.44.

N-[(*tert*-Butyloxy)carbonyl]-*trans*-3-*n*-propyl-D-proline (14b). Amide **13b** (>99% diastereomeric purity) was converted similarly to **14b**: mp 90–92 °C (hexane); $[\alpha]^{24}_D$ = +43.2° (*c* 1.0, $CHCl_3$).

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Registry No. **1a**, 123724-21-0; **1b**, 123724-22-1; **2a**, 3005-63-8; **2b**, 123724-23-2; **4**, 123724-24-3; *cis*-**5**, 123807-01-2; *trans*-**5**, 123807-02-3; **7**, 123807-03-4; **8**, 123724-25-4; *cis*-**9**, 123724-26-5; *trans*-**9**, 123724-27-6; **11**, 123877-36-1; **12a**, 123724-28-7; **12b**, 123724-29-8; **13a**, 123724-30-1; **13b**, 123724-31-2; **14a**, 123724-32-3; **14b**, 123724-33-4; (−)-MTPA-Cl, 39637-99-5; (*S*)-(−)-PhCH(Me)NH_2, 2627-86-3; (*S*)-(+)-HOOCCH(Ph)CH_2COOH, 4036-30-0; AcNHCH(COOEt)$_2$, 1068-90-2; (*E*)-H_3CCHCHCH$_2$$CH_2$$CH_3$, 6728-26-3; (*S*)-(−)-PhCH(Me)NH_2·HCl, 17279-30-0; D-*trans*-3-phenylproline, 118758-50-2; (*S*)-(+)-3-phenylpyrrolidine, 62624-46-8; (*S*)-(*R**,*R**)-*N*-[[α-methoxy-α-(trifluoromethyl)phenyl]-acetyl]-3-phenylpyrrolidine, 123724-34-5; (*S*)-*N*-benzyl-3-phenylpyrrolidine, 59349-74-5; (*R*)-3-phenylpyrrolidine, 61586-46-7; (±)-*cis*-3-*n*-propylproline hydrochloride, 123807-04-5; (±)-*trans*-3-*n*-propylproline hydrochloride, 123807-05-6; (±)-*cis*-3-*n*-propylproline methyl ester hydrochloride, 123724-35-6; (±)-*trans*-3-*n*-propylproline methyl ester hydrochloride, 123724-36-7.

Supplementary Material Available: List of positional and anisotropic thermal parameters of non-hydrogen atoms, positional and thermal parameters of hydrogen atoms, and bond distances and bond angles for compound **12b** (5 pages). Ordering information can be found on any current masthead page.

Urocanic Acid Photobiology. Identification and Characterization of the Major Photoadducts Formed between Urocanic Acid and Thymidine[1]

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Two products (adducts I and II), formed under conditions known to photochemically bind urocanic acid (UA) covalently to DNA, have been isolated from the photolysis of UA and thymidine. The gross structural features of the adducts were determined by using photolytic cleavage, mass spectrometry, UV absorption spectroscopy, ^1H and ^{13}C NMR, and ^1H–^{13}C correlation spectroscopy (COLOC). The regiochemistries were assigned on the basis of the pH dependence of hydrogen chemical shifts. The relative stereochemistries were determined by using nuclear Overhauser effects and spin–lattice relaxation times. All of these data support the assignment of the adducts as diastereomerically related cyclobutane structures resulting from 2 + 2 cycloaddition of the UA acrylic acid and thymidine double bonds.

Urocanic acid (2-propenoic acid, 3-(1*H*-imidazol-4-yl)-, UA, Figure 1) is a major component of the stratum corneum and recently has been a subject of intense photobiological interest.[2] Early studies hypothesized the biological role of UA as both a natural sunscreen and a photoprotecting agent against UV damage to DNA.[2] It is a major absorber of UV light in the skin and has been found to undergo efficient E/Z isomerization as its primary, unimolecular photochemical reaction.[2] UA is also quite reactive with singlet oxygen[2] and has been shown to photolytically generate superoxide.[3] However, more recent work has emphasized the potential deleterious effects of UA when exposed to UV light and has focused on the direct photochemical interaction of UA and biologically important components. Examples are the UA-sensitized photoinactivation of bacteriophage G4 single-stranded DNA[4] and the photochemical incorporation of UA into bovine serum albumin.[5] It has been determined that UA photochemically binds to native calf thymus DNA when these are irradiated at $\lambda > 270$ nm, with some evidence that one of the bases responsible for the photochemical incorporation is thymidine.[6] In addition, two independent studies have found that irradiated samples of (*E*)-UA can cause immune suppression in mice (there is good evidence that the causative agent is (*Z*)-UA)[7] and may increase the animals' susceptibility to cancer.[8] The results of these studies suggest that UA photochemistry may be important to the phenomenon of photocarcinogenesis. The present

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REVIEW ARTICLE

The Anticoagulant and Antithrombotic Properties of Hirudins

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Introduction

The anticoagulant properties of an extract of leech heads were recognised as early as 1884 but it was not until the 1950's that the activity, named hirudin, was isolated and characterised as a polypeptide (1). Hirudin from the leech *Hirudo medicinalis* is the most potent and specific thrombin inhibitor known. The purification and structural characterisation of natural hirudins have been extensively studied (2–5) and the primary structures of three variants designated HV1, HV2, and HV3 have been determined. More recently additional isoinhibitors were isolated from the leech with strong homology to the three known forms (5). Hirudin isoinhibitors have a single polypeptide chain of 65 or 66 amino acid residues, 6 cysteine residues and chemical molecular weights of approximately 7,000 daltons. The N-terminal five amino acid residues are hydrophobic, followed by the core region linked through three disulfide bridges. The C-terminal region is rich in acidic residues which are important for the binding to the recognition site of thrombin in the complex. In the natural molecules, the tyrosine at position 63 has been postribosomally modified to a tyrosine-0-sulphate. Fig. 1 shows sequence alignment of the three major variants and their high degree of homology with only 13 variable positions.

Cloning and Microbial Expression

Considerable progress has been made in the past 5 years by several laboratories in the cloning, expression and characterisation of biologically active, recombinant hirudins from *E. coli* and subsequently from *Saccharomyces cerevisiae* (6) or *Bacillus subtilis* (Mitsui Toatsu Chemicals). In one case, heterologous expression of recombinant HV1 (Fig. 1) in high yield has been achieved from *S. cerevisiae*. The gene coding for HV1 was synthesised in vitro on the basis of preferred *E. coli* and *S. cerevisiae* codons. Both genes were expressed in yeast under the control of the 198 base pair long constitutive GAPFL promoter. Transformed yeast strains actively secrete recombinant HV1 (CGP 39393) in the desulphated form into the fermentation broth. The polypeptide can be highly purified from the broth to homogeneity by adsorption on macroreticular resins, ion exchange chromatography and gel filtration (7).

Harvey et al. (3) isolated cloned cDNA encoding hirudin variant 2 from leech tissue (Fig. 1) and expressed it in *E. coli* under the control of the lambda P_L promoter yielding biologically active product. Recombinant HV2 and more recently recombinant HV2 lys47 were subsequently expressed in *Saccharomyces cerevisiae* with improved yields using a secretory system under the control of the α pheromone prepro sequence (MFα1). The major activity peak isolated from yeast was that predicted from the cDNA as judged by molecular mass analysis (FAB-MS), N-terminal sequencing of tryptic peptides and other physicochemical characterisations (8). The recombinant molecules described to date all lack the sulphate group on tyrosine-63 and are hence designated desulphatohirudins.

Three Dimensional Structure Analysis

The biological activity of recombinant hirudin strictly depends on the correct tertiary structure and the correct connection of the S-S bridges (Cys^6-Cys^{14}; Cys^{16}-Cys^{28}; Cys^{22}-Cys^{39}). It has been demonstrated by nuclear magnetic resonance spectroscopy (2D-NMR), that recombinant hirudin folds and is secreted from yeast in the same conformation as authentic hirudin from the leech (9).

Models of the mechanism of thrombin inhibition with hirudin have been proposed based on the involvement of multiple sites on both molecules. The N-terminal alpha amino group of hirudin HV1 and the hydrophobic nature of the two N-terminal valine residues are essential for its interaction with thrombin. It has been shown that acetylation or addition of a single amino acid to the N-terminus resulted in a marked increase in the Ki (10). Synthetic C-terminal hirudin-derived peptides form complexes with thrombin shielding accessible lysine residues of the enzyme. The data demonstrate that the structural elements of hirudin which bind to the fibrinogen recognition site of thrombin are located within the C-terminal segment of the inhibitor (11, 12).

Very recently the crystallisation of the thrombin-hirudin complex has been achieved with both the HV1 and HV2 variants and the three-dimensional structure of the complexes elucidated by X-ray analysis (13, 14). These studies show that hirudin binds to alpha thrombin by a novel mechanism for serine protease inhibitors. The tight binding of hirudin to thrombin appears to be achieved by interactions that occur both in the active site of thrombin as well as at binding sites distant from it confirming previous findings from the site-directed mutagenesis (10) and chemical modification studies (11).

Biochemical and Haematological Properties

Hirudin inactivates thrombin by blocking the substrate binding site in a 1:1 stoichiometric complex. The association rate between thrombin and hirudin is faster than that between thrombin and its substrate fibrinogen and the Ki values have been reported to be 0.02 pM for natural hirudin HV1 (15) and approximately ten times higher for the recombinant desulphated product (CGP 39393) (Table 1). All hirudin variants appear to have the same high potency as inhibitors of thrombin. Recently the three

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      1         10         20         30         40         50         60
HV1   VVYTDCTESG QNLCLCEGSN VCGQGNKCIL GSDGEKNQCV TGEGTPKPQS HNDGDFEEIP EE YLQ
HV2   ITYTDCTESG QNLCLCEGSN VCGKGNKCIL GSNGKGNQCV TGEGTPNPES HNNGDFEEIP EE YLQ
HV3   ITYTDCTESG QNLCLCEGSN VCGKGNKCIL GSQGKDNQCV TGEGTPKPQS HNQGDFEPIP EDAYDE
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Fig. 1 Amino acid sequence comparison of hirudin isoinhibitors HV1 (2, 6), HV2 (3) and HV3 (4). Amino acid residues are in the single letter code

hirudin variants, produced by gene technology (7, 8), have been tested and compared as described by Braun et al. (15). The kinetic constants for inhibition of thrombin by the three desulphated forms are within a factor of three of each other (Stone S R, 1989; unpublished).

It seems widely accepted that hirudins are specific inhibitors of thrombin but great care needs to be exercised in making use of such an interpretation. Now that hirudins have become available in sufficient quantities it is possible to investigate the effects on a wider range of enzymes and other biological mechanisms. Before the advent of recombinant hirudins the polypeptide was extracted from leeches. This resulted in not only a heterogeneous mixture of hirudin-like molecules with a variety of different amino acid sequences but there was also the risk of contamination with other protease inhibitors that have different specificities (16). Thus early work on the specificity should be interpreted with caution unless the purity of the hirudin preparation is assured. Pure recombinant desulphatohirudin HV1, at μM concentrations, has no effect on the ability of factor Xa, plasmin, kallikrein, trypsin or chymotrypsin to cleave chromogenic substrates nor is there any effect on the classical or alternative pathways of complement activation (17). In contrast, the effect on thrombin occurs in the sub picomolar range (15). In a recent study however, recombinant desulphatohirudin HV1 has been shown to displace factor Xa from the prothrombinase complex bound to cultured endothelial cells. Inhibition of prothrombin activation by this mechanism would provide an additional anticoagulant action (18). It is uncertain whether this has any significance in vivo.

Table 1 Biochemical constants for recombinant desulphatohirudin HV1

Parameter			Ref
Specific activity[1]	12,000	ATU/mg	(17)
Inhibition constant (Ki)	0.23	pmole/l	(15)
Association rate (Kon)	1.37	$molar^{-1}\ s^{-1}$	(15)
Concentration to double APTT	0.1	μmole/l	(17)
Concentration to double PT	0.8	μmole/l	(17)

[1] This is an approximate figure based on a fibrinogen clotting assay. Differences in the assay method and in the thrombin standard used have resulted in slightly different figures.

Table 2 Pharmacokinetic parameters of hirudin in healthy volunteers after a single i.v. administration of 1,000 ATU/kg (21, 36) or 0.1 mg/kg (27)[1]

	Markwardt 1984	Bichler 1988	Markwardt 1988
Source	Natural	Natural	Recombinant
$t_{1/2}$, α (h)	0.15	0.15	0.15
$t_{1/2}$, β (h)	0.84	1.08	0.91
V_d (l)	12.9	17.2	8.9
Cl_t (ml/min)	230	187	168
Cl_r (ml/min)	99.5	80	58.2
Reference	(36)	(21)	(27)

[1] These doses are approximately equivalent.

Since they bind tightly to the active site, hirudins are able to inhibit all of the effects of thrombin that are caused by its proteolytic activity. Thus, cleavage of fibrinopeptides from and clotting of fibrinogen, cleavage of small chromogenic substrates, activation of factors V and VIII are all inhibited as is the stimulation of a number of cell types including platelets (19). The affinity of hirudins is in fact high enough to cause it to displace thrombin from cellular receptors. In addition, the binding of thrombin to thrombomodulin is inhibited which may result in reduced activation of protein C (20). Hirudin thus provides a unique opportunity to unravel the complex physiological and pathological role of thrombin.

By virtue of the effect on thrombin, hirudins increase the clotting times of plasma in vitro. Thrombin time is particularly sensitive and the concentration that is required to for example double the clotting time depends on the thrombin concentration that is used to induce clotting. The dose response curve for prolongation of the activated partial thromboplastin time (APTT) is shallow up to a hirudin concentration of 1–2 μM. Similarly the prothrombin (PT) is prolonged but higher concentrations are required (17) (Table 1).

Following hirudin administration to man and laboratory animals there is close correlation between the clotting times and plasma hirudin concentration measured by a radioimmunobioassay (21), ELISA assay and thrombin inhibition (Ambler J 1989, Close P 1989; unpublished results). These quantifiable effects on simple, reproducible and commonly used laboratory clotting tests have enabled a number of investigators to monitor the plasma concentration of active hirudin in order to measure its pharmacokinetic and pharmacodynamic effects (21–23).

Antithrombotic Effects

Studies of the antithrombotic effect of hirudins in man are limited at present but a considerable body of data is available from animal models. Extensive studies in a range of models from disseminated intravascular coagulation through venous thrombosis to arterial thrombosis, which share many similarities with human disease have been carried out in order to characterise the antithrombotic efficacy.

Disseminated intravascular coagulation can be induced in animals either by administration of active coagulation factors or by eliciting a septicaemia-like syndrome by administration of endotoxin. Markwardt has shown that infusions of recombinant hirudin variant 1 between 0.03 and 0.15 mg $kg^{-1}\ h^{-1}$ inhibit the appearance of radiolabelled fibrin in the lungs of rats given an infusion of thrombokinase (22). Similarly an infusion of 0.05 mg $kg^{-1}\ h^{-1}$ of a natural hirudin preparation reduced the loss of circulating fibrinogen and platelets caused by thrombin infusion (24). In rabbits natural hirudin reduced the loss of platelets and fibrinogen and corrected the acute renal failure caused by administration of endotoxin (25). It is notable that in the studies where both parameters were measured, the effect on fibrinogen was more marked than that on platelets and it has been suggested

that this is a result of the lower affinity of thrombin for fibrinogen than for its receptor on platelets (26).

The relevance of these models to human disease is supported by the fact that in a single patient with a chronic disseminated intravascular coagulation associated with Kasabach-Merrit Syndrome, subcutaneous doses of a recombinant hirudin partially restored the low circulating platelet count and fibrinogen concentration to more normal levels for the period in which the hirudin was in the circulation (27).

Many animal models have been designed to mimic the deep venous thrombosis that is frequently found in patients who have undergone orthopaedic or other major surgery. In such models a hypercoagulable state is induced by the administration of activated coagulation factors or serum followed by a period of stasis in the vein in which the thrombus is to be formed. The thrombus can then be weighed or assessed visually. The thrombus is red in colour and has the appearance of clotted blood when examined microscopically. That is, there is no enrichment of any particular component of the blood. Hirudin can completely inhibit thrombosis in such models. For example, using recombinant HV1, dose dependent inhibition was achieved in the range of 0.03–0.20 mg kg^{-1} h^{-1} by intravenous infusion in a model in the rat where contact-activated serum was used to induce hypercoagulability (22). Similarly subcutaneous doses of recombinant HV1 in the range of 0.3–1.0 mg/kg inhibited thrombosis when tissue thromboplastin was used (23). Thus as expected of an anticoagulant, hirudin potently and effectively inhibits thrombosis where the mechanism of induction is primarily activation of the coagulation system and the production of fibrin. Of more recent interest is the effect in models where platelet involvement plays a major role.

It has been known for some years that anticoagulants like warfarin and heparin have inhibitory effects on the formation of platelet-rich thrombi (28). Early models used foreign surfaces of various degrees of thrombogenicity that are placed in blood flowing through an extracorporeal circuit in vivo and in this respect they share some features of the situation where blood is passed over surfaces like dialyser membranes or prosthetic devices in man. When such models have been more fully characterised, it is clear that there is considerable recruitment of platelets from the circulation such that a large proportion of the thrombus is platelet aggregates. Inhibitors of platelet function generally have some inhibitory effect whereas they are usually inactive in models of venous thrombosis (29). Hirudins inhibit thrombosis on these foreign surfaces and are capable of complete inhibition whether the thrombus formation is measured gravimetrically or as the time taken for occlusion of the extracorporeal circuit. Effective doses vary widely depending on the thrombogenicity of the surface used but in all cases they are higher than those required to inhibit venous thrombosis and this again probably reflects the different sensitivities of fibrinogen cleavage and platelet aggregation to thrombin (22, 23).

The formation of thrombi on damaged arteries is especially dependent on the involvement of platelets. In animals it is difficult to induce a reproducible injury to an arterial wall without recourse to quite severe electrical or chemical injury and then it is by no means certain that the injury caused resembles that which occurs in man. Thrombus formation has been assessed by changes in blood flow, occlusion or by the incorporation of radiolabelled fibrin or platelets. Hirudins are effective in such models and the doses required are higher than those required to inhibit venous thrombosis (22, 30).

Probably of greater relevance to the clinic are those models where vascular damage is caused by mechanical means. When an angioplasty balloon is inflated in the carotid artery of the pig, very similar morphology to that observed after similar procedures in man can be demonstrated (31). There is considerable damage to the endothelial cells and in about 50% of the animals there are deep tears in the intima that penetrate the internal elastic lamina into the media. One reservation that should be expressed about such a model is that the artery is normal and that in the clinic angioplasty is usually performed on severely atherosclerotic vessels. Nevertheless a large thrombus forms at the site of damage and it comprises mainly platelets (31). Intravenous infusions of recombinant hirudin HV1 (1 mg kg^{-1} h^{-1}) completely obliterated thrombus formation and reduced the platelet incorporation by about 80% such that only a very thin layer of platelets remained (32, 33). In the rat, a model of arterial thrombosis where mechanical damage is elicited to the dorsal aorta by application of a pressure clamp has been developed (34). The resultant morphology is similar to that described above for the pig with large deposits of platelets attached to the tears in the vessel wall. Thrombosis was evaluated by the incorporation of radiolabelled platelets and fibrinogen on to the injured segment. Recombinant hirudin HV1 dose dependently inhibited both platelet and fibrin deposition, at subcutaneous doses in the range of 1–10 mg/kg, to more than 75% (34).

The most notable feature of this wide spectrum of antithrombotic activity of hirudins is that, as the thrombus forms in more rapidly flowing blood and platelets become a more dominant feature, the concentration of hirudin required to inhibit thrombus formation increases. From venous thrombosis models to arterial thrombosis models this increase amounts to about a factor of ten. As we begin to look forward into the likely effects of hirudins in the clinic the question becomes whether such high plasma concentrations can be achieved in patients without some loss of safety. The greatest danger in the use of high doses of anticoagulants is the risk of bleeding and it is no surprise that in animal models, hirudins do increase the bleeding time (22, 33). However, bleeding in animal models such as that caused by transection of the rat or mouse tail or by incisions made by a template device are unlike the cerebral or surgical bleeding that is the major risk in man. It is consequently impossible to draw any meaningful conclusions about this therapeutic index from such studies and it will be necessary to carry out very carefully controlled studies in man in order to answer this complex question. The best answer that is available at present is to relate the therapeutic doses to their effects on standard laboratory clotting tests. In rats it is possible to achieve maximal inhibition of arterial thrombosis when the APTT is prolonged by a factor of less than 4-fold compared to controls (34) and in the pig model thrombosis is maximally inhibited when the APTT is 2.5–3.5 times the control (32, 33). Whether this is indeed tolerable in man when hirudin is used will be answered by the present and future clinical trials.

Pharmacokinetics and Pharmacodynamics

The pharmacokinetics of both natural and recombinant hirudins in animals has been well reviewed by Markwardt (1, 19). Essentially, the volume of distribution indicates that hirudins distribute rapidly into the extracellular space and are cleared from the plasma compartment with a half life of around 1 h depending on the species. In dogs, a high proportion of the administered dose appears intact in the urine. In addition, since the total clearance closely approximates the renal clearance and since clearance is undetectable in nephrectomised animals it appears that this is the sole route in dogs (19).

In rats the situation is less clear. Nephrectomised rats do not appear to clear recombinant hirudin from the plasma (Butler K, 1988, unpublished results) so it is reasonable to assume that the kidney is the major organ for clearance from the plasma in this species as well. However, only about 25% of radiolabel from intravenously administered ^{125}I iododesulphatohirudin appears in

the urine so it is therefore likely that some hirudin remains in, and is presumably metabolised by, the kidneys in this species (38). This notion is supported, but not proven, by the fact that radioactivity from iododesulphatohirudin becomes concentrated in the kidneys of entire animals 5 h after i.v. administration (38).

The situation in man is very similar to that in the rat (Table 2). Plasma concentrations decline in a biphasic manner. The first phase can be interpreted as being the distribution whilst the second phase represents clearance and results in a plasma half-life of about 1 h after i.v. administration. The observed duration of action can be prolonged by using the subcutaneous route where bioavailability is high (85%, ref. 21) or the intramuscular route (27). A proportion appears unchanged in the urine and this amount increases with the dose but unlike that in the dog, the total clearance rate cannot be fully accounted for by the fraction appearing in the urine (Table 2) and it is likely that some other route of elimination, possibly metabolism in the kidney, exists in man.

Safety Issues

As discussed above, a major physiological role of thrombin is likely to be in haemostasis. Inhibition of this enzyme by potent agents like hirudins could therefore lead to an increased loss of blood from leaks in the vascular tree. The mechanisms of haemostasis differ depending on the site and hence morphology of the injury, the flow rate and probably also the species. Hirudins do have effects on bleeding in some animal models (22, 33, 37) but it is not at all clear whether this indicates that there will be an effect in man.

The bleeding time has been measured in three separate human studies and a small prolongation was observed in some volunteers in two of these when the hirudin plasma level was high (27, 35, 39). However since the test methods have considerable variability and their relevance to clinical bleeding is unknown, no conclusion can therefore be drawn yet about the potential safety margin of hirudin and it will be necessary to gain more clinical experience before it is possible to judge this important issue.

Since hirudin is a polypeptide from a non-human source, there may be concern about its potential for immunogenicity or possible allergenicity. This has been addressed in all the human studies to date and no evidence of such phenomena was observed from antibody measurements or side effect assessment in any of the volunteers whether they had received either one or two administrations (21, 27, 36, 39). The risk of allergenicity therefore appears to be low.

Hirudins have provided us with a new concept in anticoagulation. They appear to act primarily through inhibition of thrombin and have a wide spectrum of antithrombotic effects in animal models. In man they appear to be well tolerated and have no dramatic effect on tests for bleeding. They appear to be non-immunogenic in human subjects and have no observable side effects. It will be of great interest to see if they have significant advantages over the existing therapy for thrombotic disease. In addition they should enable us to gain an important insight into the physiological and pathological role of a potent platelet agonist and the final enzyme in the coagulation pathways, thrombin.

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